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DANIEL MELNICK AND BERNARD L. OSER

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DANIEL MELNICK[b] AND BERNARD L. OSER[c]

The concept of food protein as N x 6.25 should be extended to include a consideration of both the functional and nutritive properties of the protein. The functional properties may be evaluated by the use of standardized procedures for determining the degree of protein denaturation; the nutritive properties, by determining the amino acid composition of the protein and its susceptibility to enzymic digestion. For optimal utilization of food proteins all essential amino acids must not only be available for absorption but must also be liberated during digestion in vivo at rates permitting effective mutual supplementation.

Since the major portion of this report deals with the susceptibility of protein to enzymic digestion as a factor in nutrition, the title of this paper might have been "The Influence of Heat-Processing of Protein on the Availability of the Amino Acids" in keeping with the subject of this symposium. However, the functional properties of protein, also readily modified by heat-processing, affect appearance and palatability of the end-product and therefore cannot be dissociated from the nutritive properties of the protein. Food must be consumed in order to make its nutrient contribution.

It is not uncommon for a given food material to be processed in different ways without a change in its protein content or in the amino acid composition of the protein complex; yet these foods cannot be used interchangeably without modifying the quality of the end-product. Many food technologists, aware of the limitations of their raw materials, usually strive to attain food formulations which give optimal performance, the functional properties of the protein receiving primary, if not exclusive, consideration. On the other hand, the nutritionist concerns himself with the influence of processing on the nutritive value of the protein, striving to avoid destruction of the essential amino acids and to attain a product free from factors which might interfere with the availability of the amino acids. Ease of manufacture, appearance, palatability, stability, and ready utilization of the end-product, however, are important considerations and cannot be sacrificed even for optimal nutritive value. The ultimate aim, of course, should be to obtain a food or a blend of food materials in which both functional and nutritive properties of the protein are at a maximum. However, this can be realized only in a few instances.

The experience of manufacturers of soy products will serve as an excellent illustration of the problem to be presented. Soy flour is used principally as (a) a wheat flour extender, (b) as a source of "tofu," an oriental main dish prepared by forming a curd from the water-extractable soybean protein, and (c) as a major ingredient in low-cost food preparations such as soup mixes. For the first two purposes it is essential that the protein in the product be in an undenatured form so that the functional (utility) properties will be unimpaired. However, in those food products consumed after minimal heating at atmospheric pressure and in which the functional properties of the protein are not critical, the soy product should be adequately processed to increase the biological value of the protein to a maximum. In raw or insufficiently heat-processed soy products an anti-tryptic factor is present *(8, 28)* which interferes with the digestibility of the soy protein; excessive heat-processing effects a reduction in the nutritive value of the protein *(79, 62, 10, 20, 25)*. The soy industry has been aware of this situation and has attempted to manufacture products under standardized conditions to serve specific functions. A soy flour intended for use in bread may not be satisfactory for inclusion in a soup mix and vice versa. The group at the Quartermaster Food and Container Institute has prepared a specification for the procurement of soy products which unfortunately fails to recognize these differences. Simple objective test methods are not available at the present time for inclusion in a specification to justify setting specific requirements for the two kinds of soy flour desired; namely, for baking bread and curd formation, or for soups, nährmittel, etc. A collaborative research project with the soy industry is now under way for the purpose of incorporating in the specifications suitable methods of test to insure that flour required for a specific purpose is procured. Too frequently have reports been received from overseas areas severely criticising the soy products furnished. Heat-processed soy meal containing practically no water-extractable protein has been furnished to the civilian populace in the Orient, whereas low heat-processed soy flours have been shipped to Germany for inclusion in soup mixes and food combinations which are consumed either as such or after minimal heating.

The Functional Properties of Protein in Food Materials

In Table 1 are shown five food products subjected to heat-processing with the view toward obtaining products whose protein components exhibit desirable functional

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[a] This paper from the Quartermaster Food and Container Institute for the Armed Forces, as one of the participating laboratories, has been assigned Number 189 in the series of papers approved for publication. The paper was presented by the senior author in a symposium on "Amino Acids" before the Institute of Food Technologists, Eighth Annual Convention, Philadelphia, Pa., June 9, 1948. The views or conclusions contained in this report are those of the authors. They are not to be construed as necesssarily reflecting the views or indorsement of the Department of the Army.

[b] Quartermaster Food and Container Institute for the Armed Forces, Chicago, Ill. The experimental work on the nutritive properties of proteins was carried out while the senior author was associated with Food Research Laboratories, Inc., Long Island City, N. Y. Certain phases of this work have been reported elsewhere *(60b, 52, 61)*. Studies on both the functional and nutritive properties of proteins are continuing at the Quartermaster Food and Container Institute for the Armed Forces.

[c] Food Research Laboratories, Inc., Long Island City, N. Y.

TABLE 1

Influence of Heat Processing on the Functional Properties of Protein

Protein Source	Heat Processing	Resulting Product
Soybean	100°-135° C. for 10-0.1 min.	Slightly denatured protein (50-65% extractable) enzyme (diastase and lipoxidase) inactivated; used for curd formation in main dish preparations, in bread manufacture, in sausages, and in alimentary pastes.
Egg	Preheated to 60° C., spray drying to 2.0% moisture content, temperature $<65°$ C.	Slightly denatured protein; used to form a coagulum in main dish preparations, for imparting proper body and structure to baked products, spaghetti, etc., for emulsifying properties.
Yeast	28-30° C. to 8% moisture content.	Enzymes in intact cells still active; used as a fermentation agent in baking industry.
Flour	55-80° C. for approx. 1 min.	Accelerated maturation, replacing agene process, with the result that concentration of free sulfhydril compounds is reduced without impairment of the diastatic activity; used in baking industry.
Milk	90-92° C., 15 min.	Denatured protein, enzymes inactivated, low in volatile sulfhydril groups; used in bread manufacture.
	70-72° C., 15 sec.	Slightly denatured protein, which redissolves in preparing beverage.
	61-62° C., 30 min.	Slightly denatured protein in frozen pasteurized milk.

properties. Additional materials could have been included, but those listed will suffice for this discussion. Excessive heat-processing (or minimal heating in the case of the milk powder employed for baking purposes) alters the character of the products to such an extent that they can no longer be employed for the purposes intended.

Rather than depend solely upon subjective utility tests for rating food materials in which the functional properties of the protein are susceptible to change during processing, it is suggested that precise, objective tests of the extent of protein denaturation be employed. Of course, in each case correlation between the utility and biochemical tests has to be established. Various laboratory procedures for ascertaining the degree of protein denaturation are already being employed in the laboratories of the food industries. Thus in the case of soy and egg protein, measurements of the solubility index are usually made. The results of these tests characterize the functional properties of the product at the time the material is freshly prepared; these, however, do not reflect the potentialities of the protein to undergo further changes during the subsequent storage periods, with a resulting decrease in solubility. It would seem desirable to supplement the solubility measurement with tests to ascertain whether or not the salting-out characteristics of the protein have been altered. Denaturation of coagulable protein can occur without precipitation *(51)*. Since the salting-out characteristics of proteins change under conditions inducing incipient denaturation, a quantitative salting-out test should serve as a better indicator of the actual degree of denaturation effected, than the simple solubility measurement, and of the tendency of the protein to become insoluble during prolonged storage. Harland and Ashworth *(29)* have used the salting-out behavior of the whey protein in dried milk for purposes of classifying dried skim milk samples with respect to baking qualities. In this case protein denaturation is desired. At the Institute a precise objective protein precipitation technique has been evolved for rating the potentialities of soy preparations for curd formation. This same procedure may prove to be useful in rating soy flours for use in bread manufacture.

Influence of Heat-Processing on the Nutritive Value of the Protein

The above title constitutes the major theme of this discussion. Three factors, broadly speaking, determine the nutritive value of a food as a source of protein. These are (a) the quantity of protein expressed in terms of total solids content, (b) the essential amino acid composition of the protein, and (c) the availability of the amino acids for tissue protein synthesis.

It is incorrect to compare two food products on the basis of their protein content alone without reference to their total solids content. Reference is sometimes made to the protein value of a cereal or leguminous food relative to that of meat. In terms of total solids, materials like oats or soybeans furnish much less protein than meat, even though their net protein content may be the same or even greater.

The chief consideration in the selection of dietary proteins is their content of indispensable amino acids, since proteins can be no better than the biological sum of their amino acids. Animal proteins have greater biological value than vegetable proteins because they contain a greater number of essential amino acids in quantities of nutritional significance. The essential amino acids, however, must not only be present but must be readily available for absorption and utilization by the animal organism.

Papers have appeared demonstrating with a number of species that the protein in properly heat-processed soy products has a much higher biological value than that of the raw bean *(60, 55, 34, 35, 79, 33, 62, 32, 2, 15, 54, 20, 25, 46, 77)*. Supplementation with methionine eliminates in large part the discrepancy between the two products *(32, 2, 54, 20, 11, 68)*. In addition to soybeans, many other leguminous proteins seem to improve in nutritive value after the application of heat *(38, 75, 22, 76, 23, 21, 67)*. On the other hand, excessive heating has been shown to impair the biological value of proteins. The adverse effects of heat on milk proteins (particularly casein) *(56, 27)*, on edestin *(73)*, on blood globin *(14)*, and on the proteins in cereals *(56, 44, 58, 71, 43, 64)*, meat *(57, 69, 30)*, and fish products *(50, 66, 78)*, in soybean *(79, 62, 10, 20, 25)*, and other legumes *(83, 21, 59, 67)*, in coconut

meal *(54)*, and in a baking formulation *(6)* have been reported. In practically every case supplementation of the ration with lysine effected substantial if not complete correction of the nutritional deficiency.

Storage of foods over extended periods of time appears to cause impairment in the nutritive value of the protein similar to that noted following excessive heat-processing. This has been observed in studies with dried skim milk *(36)*, with soy meal *(41)*, and with the cereal grains *(41, 42, 40)*.

Throughout the papers indicated above, data are presented demonstrating that heat-processing of foods has a profound effect on the nutritive value of the protein without affecting the protein content, the essential amino acid composition, or the degree of protein digestibility *in vivo*. Despite the fact that methionine is the principal limiting amino acid in raw foods, whose protein value can be improved by heat-processing, and that lysine plays a corresponding role in the case of excessively heated proteins, the contents of these amino acids are not materially affected by the processing techniques. This had led to the conclusion that these amino acids in the ineffective proteins are not biologically available *(84, 17, 7)*. In the case of lysine it has been postulated *(17, 7)* that a new peptide linkage forms as a result of heat-processing involving the ϵ-amino group of lysine and the free carboxyl group of a dicarboxylic amino acid and that this complex is resistant to enzymic digestion.

In the present report data will be presented demonstrating with heat-processed proteins that the *rate* of enzymic liberation of the amino acids, rather than *degree* of amino acid availability, is of critical importance in determining the efficiency with which the absorbed amino acids are retained for tissue protein synthesis. In studies of amino acid supplementation of deficient amino acid mixtures *(18, 26, 9)* or of an inadequate ration *(4)*, or of proteins supplementing each other *(37)*, time intervals have proved to be exceedingly important; as the time interval between feeding the dietary components is reduced more effective biological responses are obtained. As a working hypothesis in the present studies, it has been postulated that for optimal utilization of protein all essential amino acids must not only be available for absorption but must be liberated during digestion *in vivo* at rates permitting mutual supplementation, and that heat-processing influences the relative rate of liberation of the amino acids. That amino acids are liberated during digestion at different rates has previously been reported *(53)*, but the nutritional implications of such observations have been largely neglected. Other factors, such as the presence in foods of naturally occurring toxic agents *(45)*, vitamin-amino acid interrelationships *(53b)*, or the necessity for the animal organism to absorb amino acids in certain peptide linkages *(80, 81, 82)* undoubtedly also play an important role in protein and amino acid utilization.

In the early phases of the present investigations it became apparent that there would be need for a simple *in vitro* technique for estimating the susceptibility of protein, before and after heat-processing, to enzymic digestion. In this report are presented the *in vitro* digestibility method selected, the details and reliability of the improved formol titration procedure used in the *in vitro* studies, the variables requiring control in the application of the *in vitro* procedure to the determination of the susceptibility of protein to enzymic digestion, the results of the *in vitro* studies of the influence of heat-processing on protein digestibility, and the correlation of the *in vitro* findings with the results of biological and microbiological studies.

Experimental Part

Various methods are available for biological determination of the nutritional value of protein. The preferred technique is undoubtedly the nitrogen balance method of Mitchell and his associates *(53)*. The procedure affords a measure of the retention of absorbed nitrogen for tissue protein synthesis (the biological value of the protein) and at the same time gives a specific value for the percent of the dietary nitrogen that is absorbed (the degree or coefficient of protein digestibility). That the precision of the assay technique is excellent is indicated by the ease with which reproducibility can be held within $\pm$ 5 percent. However, the procedure demonstrates but does not explain why the same protein after various methods of processing shows differences in biological value even though the amino acid composition and coefficient of digestibility remain unchanged. It will be shown in this report that by supplementing the bioassay technique with an *in vitro* digestibility procedure, the probable basis for this phenomenon may be elucidated. Indeed, the method for determining the *in vitro* digestibility of a given food material, after correlation has been obtained with the bioassay has practical value in plant control of processing methods affecting the nutritional quality of the protein since it obviates the need for confirmatory bioassays.

The in vitro Digestibility Technique

The details of the *in vitro* technique for estimating susceptibility of proteins to enzymic digestion are presented in Figure 1.

A suspension or solution of the material containing six

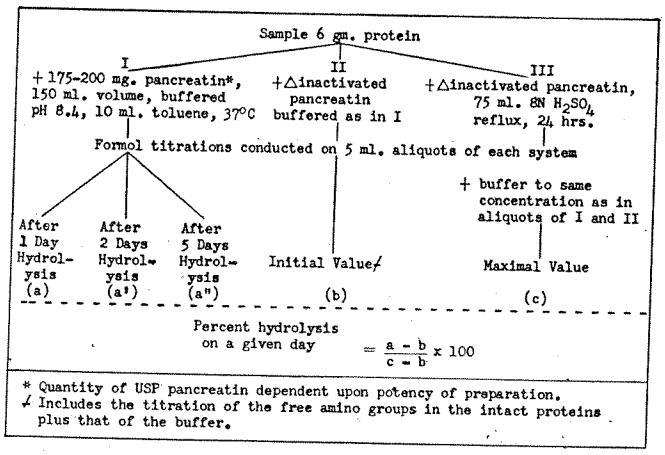


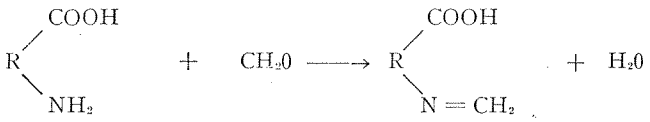
FIG. 1. *In vitro* technique for estimating susceptibility of proteins to enzymic digestion.

grams of protein is buffered [d] to pH 8.4, 175 to 200 mg. of USP pancreatin added, and the volume brought to 150 ml. Ten ml. of toluene are introduced and the mixture incubated at 37° C. Periodic formol titrations are conducted on 5 ml. aliquots of the enzymic digests. To another sample of the protein-bearing material, similarly buffered and diluted, is added the heat-inactivated pancreatin and an aliquot then taken for the formol titration. To a third sample of the test material, the sulfuric acid is added, and the suspension refluxed for 24 hours. A 5 ml. aliquot is taken, buffer added to the same concentration as in aliquots of I and II, and the material then analyzed by formol titration. In the case of III, the maximal titration value is obtained, representing ultimate hydrolysis to the amino acid stage. Thus (a — b), the increase of free amino nitrogen on a given day, over (c — b), the theoretical value for liberated amino nitrogen in a completely hydrolyzed sample, times 100 gives the percent hydrolysis of the protein. (It has proved desirable in many instances to determine the percent hydrolysis attained at intervals during the first 24-hour period). The use of sub-optimal quantities of the pancreatin permits digestion to proceed for several days at 37° C., slowing down the rate of liberation of the amino acids to such an extent that differences in digestibility of proteins may be detected. In materials containing readily digestible protein, hydrolysis is deliberately limited to approximately 50 percent. Obviously, this system does not duplicate conditions in the gastrointestinal tract. However, it constitutes a simple method for use as a routine laboratory control measure of the relative digestibility of the protein in materials undergoing heat-processing. Correlation of this simplified procedure with the results of animal assays to be presented in this report has amply justified the empirical conditions of the test.

The validity of the *in vitro* technique for determining protein susceptibility to enzymic digestion leans heavily on the reliability of the formol titration procedure.

The Formol Titration Procedure and Its Reliability

The oldest and simplest formulation [e] of the reaction between amino acids and formaldehyde is as follows:



As a result of the reaction the basic properties of the amino group are destroyed, permitting direct titration of the carboxyl group.

In carrying out the formol titration, the test solution, containing approximately 30 mg. of nitrogen, is first adjusted to pH 7.0 in a total volume of 20 ml. Two ml. of a 37 percent solution of formaldehyde, adjusted to pH 7.0, are added. This causes a decrease in the pH of the solution in most cases between 5.0 and 6.0. The solution is then titrated to pH 9.5 with standard O.1N sodium hydroxide. The formol titration as usually described is based on the use of a visual end-point, phenolphthalein being employed as the indicator. In the present procedure all pH measurements are made with the Beckman pH meter, using the glass electrode. Whereas the methods employed in the literature, almost without exception, terminate the formol titration at pH 8 to 9, it is specified here that the test solutions be titrated to pH 9.5.

The justification for this change in the procedure is evident from the titration curves in Figure 2. Here are plotted the percent of total nitrogen titrated at a given pH following the addition of the formaldehyde. It may be noted that at pH 9.5 the formol titratable nitrogen values equal the Kjeldahl nitrogen figures for ammonium chloride, for valine (a typical monoamino-monocarboxylic acid), and for glutamic acid (a typical monoamino-dicarboxylic amino acid). In the case of glutamic acid the adjustment of the solution to pH 7 neutralizes one carboxyl group so that subsequent titration, following the addition of formaldehyde, is similar to that for the monoamino-monocarboxylic acid. In the case of tryptophane and the basic amino acids (each of the latter as the hydrochloride), the formol titratable nitrogen values are lower. In valine there is one nitrogen per carboxyl group; in the others there is more than one nitrogen per carboxyl group. Thus for tryptophane containing two nitrogens the value calculated from the formol titration is found to be exactly one-half of the total Kjeldahl nitrogen; for arginine containing four nitrogens per carboxyl group the nitrogen by formol titration is exactly one-fourth; histidine with three nitrogens per carboxyl group, gives a formol titratable nitrogen value 35 percent of the total nitrogen figure. On the other hand, lysine with two nitrogens gives a value 90 percent of the Kjeldahl value. Apparently in the case of lysine the hydrochloride group still remains attached to one of the amino groups when the solution is brought to pH 7, and this as well as the carboxyl group is titrated following reaction of formaldehyde with the amino groups. The basicity of the guanidine group in arginine is probably so great that the bound hydrochloric acid is not released following neutralization to pH 7.0, addition of the formaldehyde and subsequent titration. In the case of histidine, having an isoelectric point very much closer to that of the monoamino-monocarboxylic acids than that of either lysine or arginine, little hydrochloric acid remains bound after the pH adjustment to 7.0 to complicate the formol titration. In titrating the other amino acids selected, some discrepancies between formol titratable and total nitrogen values are found but these are not excessive.

It is apparent from the results obtained that if the titrations were terminated at pH 8 or 9, appreciably

[d] The test material is suspended or dissolved in 60 ml. of a phosphate buffer solution (250 ml. of M/5 KH_2PO_4 plus 240 ml. of M/5 NaOH, diluted to 1000 ml. volume; pH 8.4), and the pH readjusted to 8.4, if necessary. Ten ml. of the buffer solution containing the pancreatin are then added and the volume brought to 150 ml. mark with water.

[e] The formation of monomeric Schiff bases in the case of aliphatic compounds may be questionable; compounds with the chemical composition of Schiff bases are more likely cyclic trimers. For an informative discussion of the reaction of formaldehyde with amino acids and proteins, reference is made to the excellent review article by French and Edsall *(24)*.

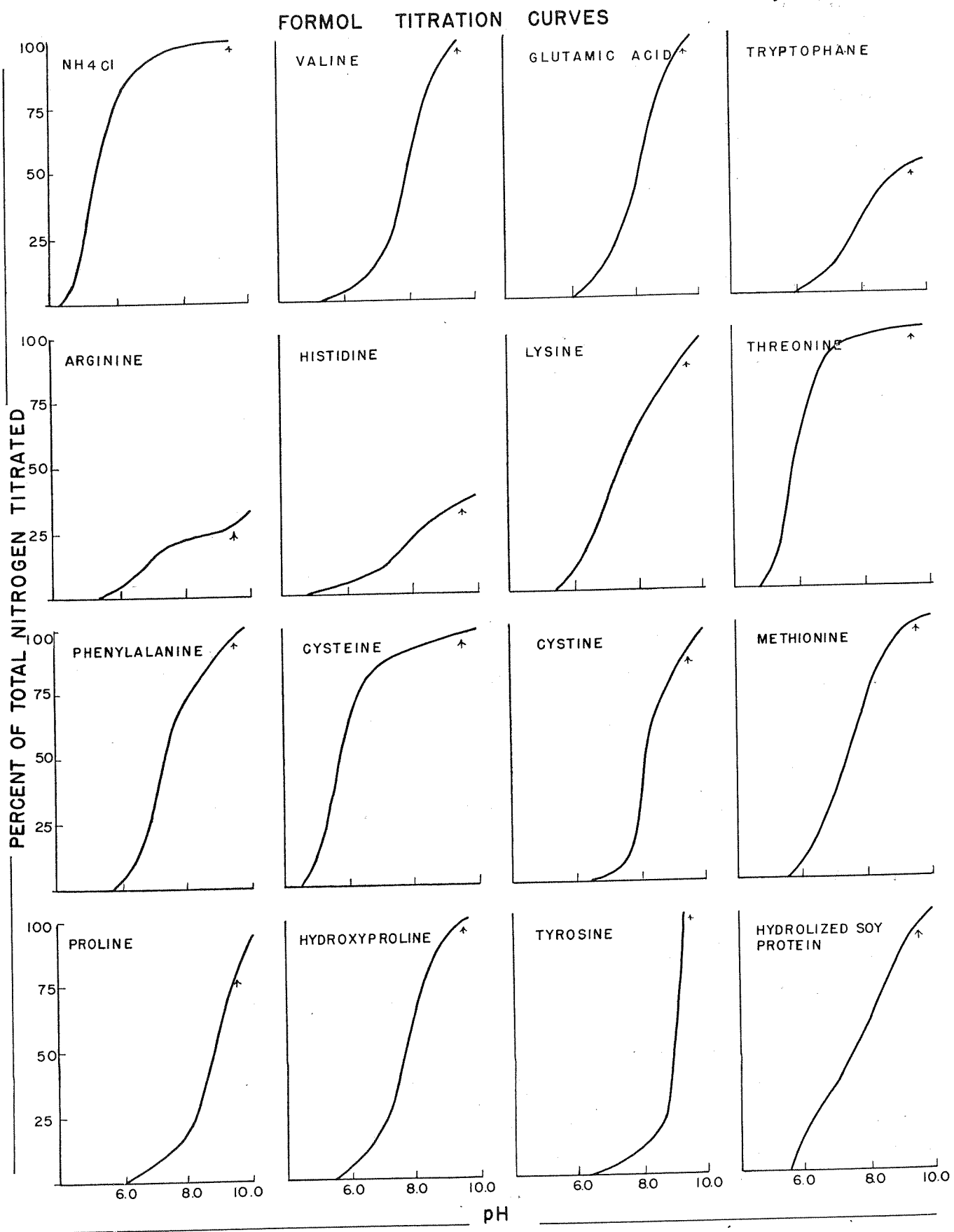


FIG. 2. Relation of formol titratable nitrogen figures at different pH values to total nitrogen content. (The arrows point to pH 95.)

greater deviations from the total nitrogen values would have been obtained. The data with the soy meal protein are of interest. They indicate how the formol titration works in actual practice. It will be noted that 94 percent of the total nitrogen is measurable by formol titration. This should be expected according to the following reasoning: The total lysine, arginine, histidine, and tryptophane content of most proteins varies between 10 and 20 percent; in soy protein they total about 14 percent. The formol titration as described actually measures somewhat more than one-half of the total nitrogen of these amino acids. Thus, 86 plus 7 or 93 percent of the theoretical value should have been obtained on titrating this protein hydrolysate.

Further justification for selecting pH 9.5 as the end-point of the formol titration may be derived from the data listed in Table 2. Eighteen food products were

TABLE 2

Justification for Selecting pH 9.5 As the End Point for the Formol Titration

Sample	Nitrogen Content		
	Kjeldahl Analysis (a)	Formol Titration* (b)	Deviation†
	percent	*percent*	*percent*
Raw Soy Meal	6.00	5.63	— 6.1
Expeller Processed Soy Meal	7.39	6.80	— 8.0
Solvent Processed Soy Flour	8.43	7.71	— 8.5
Dried Skim Milk	5.45	5.62	+ 3.2
Whole Milk	0.55	0.53	— 6.8
Casein	14.36	13.47	— 6.1
Whole Egg	1.92	1.89	— 1.7
Yeast Powder	7.40	7.40	0.0
Rice Bran Concentrate	1.41	1.41	0.0
Whole Oats	1.94	1.98	+ 2.5
Dehulled Cooked Oats	2.54	2.62	+ 3.1
Defatted Sunflower Meal	8.24	7.46	— 9.4
Whole Wheat	2.61	2.70	+ 3.4
Defatted Wheat Germ	5.54	4.67	— 15.6
Beef Steak	3.32	3.12	— 6.3
Chicken Product	2.40	2.46	+ 2.6
Liver Product	2.19	1.98	— 9.5
Whole Beef Blood	2.88	2.80	— 2.8
Average			— 3.6

*Of acid hydrolyzed sample.

†$\frac{b - a}{a} \times 100$.

subjected to Kjeldahl analysis and to formol titration according to the procedure described. The deviation of the formol titratable nitrogen from the Kjeldahl nitrogen is indicated in the last column. It will be noted that nitrogen by formol titration tends to be lower than by Kjeldahl anaylsis, being on the average about 4 percent less. From the titration curves presented in Figure 2, it is apparent that this could have been predicted.

The Control of Variables in the Application of the *in vitro* Procedure to the Determination of the Susceptibility of Proteins to Enzymic Digestion

Obviously the rate and degree of proteolysis should be dependent upon the activity of the pancreatin. In Table 3 are presented the results of tests of three soy meals and of Labco casein with fresh USP pancreatin and with a preparation which had been stored two years at 5° C. These two preparations had originally given the same formol titration values for the same test samples. It will be noted that in the case of soy meals the rate of hydrolysis is affected by the activity of the pancreatin, but only after the first day of hydrolysis. However, the relative ratings of the samples are not affected by the pancreatin employed. In any extended series of studies where it might be desirable to compare the degree of susceptibility of protein to enzymic hydrolysis, it is essential that the activity of the enzyme preparation be eliminated as a variable. This can be accomplished by standardizing each new batch of pancreatin to allow use of equivalent weights of the preparation. On the basis of the results presented here, it was decided not to employ casein as the substrate for the standardizations. Casein is so readily digestible that smaller quantities of enzyme failed to influence its rate of digestibility to the same extent as in the case of some other test materials stored in an hermetically sealed container at 0° C. A heat-processed soy meal is preferred for standardizing each batch of enzyme material.

If possible, the protein substrate subjected to the *in vitro* test for digestibility should be treated to render inactive any proteolytic enzymes that may be present. This is apparent from the illustrative data in Table 4 on raw soy meal, heat-processed soy meal, and casein. The latter was included because of the report by Warner and Polis *(74)* which claimed that all commercial casein preparations contain proteolytic enzymes. It will be noted that in the case of the raw soy, incubation of the material without the pancreatin gave rise to formol-titratable nitrogen. However, the proteolysis due to the enzymes naturally present and that due to the added pancreatin are not additive but synergistic. Thus the results of the digestitbility tests of the fat-extracted, enzyme-inactivated raw soy with added pancreatin gave values much less than would be obtained by subtracting the result of Experiment 2 from that of Experiment 1. Heat-processed soy, of course, is enzymically inactive. The tests have demonstrated no significant proteolysis of casein (Labco vitamin-free casein) incubated under the conditions described. From the data for raw soy meal it is obvious that erroneous results may be obtained for protein digestibility if the naturally present enzymes are not previously inactivated.

Pancreatin also contains a lipase capable of releasing free fatty acids which are neutralized at a pH above 7.0. Thus if a significant amount of free fatty acids were liberated in a digestion mixture, the neutralization of the

TABLE 3

*Rate and Degree of Proteolysis Dependent Upon Activity of Pancreatin**

Pancreatin	Test Sample	Degree of Hydrolysis of the Proteins		
		After 1 Day	After 2 Days	After 5 Days
		percent	*percent*	*percent*
Fresh Preparation	Soy Meal No. 1	8	15	18
	Soy Meal No. 2	12	19	28
	Soy Meal No. 3	12	19	33
	Casein	19	32	43
Stored 2 Years at 5° C.‡	Soy Meal No. 1	9	10	13
	Soy Meal No. 2	12	14	17
	Soy Meal No. 3	10	14	21
	Casein	20	31	37

*For each experiment 200 mg. of USP pancreatin was employed.

‡ Sample had been used repeatedly during this holding period, involving frequent removals from the refrigerator and exposure to the atmosphere.

TABLE 4

Influence of Naturally Occurring Proteolytic Enzymes on the In Vitro Test of Protein Digestibility

Sample (Protein Content)	Experiment		Hydrolysis of Proteins		
	No.	Description	After 1 Day	After 2 Days	After 5 Days
			percent	*percent*	*percent*
Raw Soy Meal* (38.3%)	1	Incubated with pancreatin	24	29	37
	2	Incubated without pancreatin	0	4	9
	3	Fat extracted, inactivation of enzyme at 100° C., then incubated with pancreatin	10	16	21
	4	Same as 3, but without pancreatin	0	0	0
Processed Soy Meal (49.0%)	5	Incubated with pancreatin	14	25	37
	6	Incubated without pancreatin	0	0	0
Casein (91.6%)	7	Incubated with pancreatin	19	32	43
	8	Incubated without pancreatin	0	0	0

*Digested samples subjected to ether extraction prior to formol titrations.

TABLE 5

Influence of Fat in Test Sample on the In Vitro Test of Protein Digestibility

Sample	Composition		Test	Enzymic Hydrolysis of Proteins			F. T. N.† in Acid Hydrolyzed Sample
	Protein	Fat		After 1 Day	After 2 Days	After 5 Days	
	percent	*percent*		*percent*	*percent*	*percent*	*% Kjeldahl Value*
Whole Milk	3.50	4.0	Direct	16	21	25	113
			Defatted*	20	25	28	93
Hard Boiled Egg	12.0	11.5	Direct	10	13	29	103
			Defatted*	10	14	31	98
Raw Soy Meal	38.3	21.8	Direct	9	16	20	100
			Defatted*	10	16	21	91
Expeller Processed Soy Meal	49.0	1.6	Direct	14	25	37	91
			Defatted*	14	25	37	91

* Enzymic and acid digests adjusted to pH 1, extracted with ether (residual solvent evaporated off), and then subjected to formol titrations.
† "Formol titratable nitrogen."

fatty acids might lead to erroneously high formol titration values. The significance of the fat in the test sample on the *in vitro* test of protein digestibility is presented in Table 5. The ratio of protein to fat may be calculated from the data in columns 2 and 3. When the amount of fat relative to protein is large, such as in egg or whole milk, an error may be introduced in the formol titration. The actual titration figures demonstrated that during *acid* hydrolysis of the protein-bearing materials, the fat is readily converted to free fatty acids which are then titrated. However, only negligible amounts of fat are hydrolyzed by the pancreatin under the conditions of the test, so that the formol titration values on the samples before and after ether extraction remain essentially the same. Because of the increase in apparent formol-titratable nitrogen in the acid-hydrolyzed samples, and therefore in the denominator in the fraction expressing degree of proteolysis, there appears to be a decrease in protein digestibility during enzymic digestion of samples containing a large proportion of fat. In products with approximately one-half as much fat as protein, for example the raw soy meal, no appreciable error is introduced by use of the direct formol titration. A factor responsible for the smaller-than-expected degree of interference of the fatty acids in this series is the rapidity with which formol titrations are routinely carried out. The fatty acids are insoluble in water and only by neutralization with sodium hydroxide are they brought into the aqueous phase. This involves stirring and delay after each addition of alkali. In the present series, the titration values were obtained with the same timing required for conducting formol titrations on samples free of fat, that is, about five minutes for the titration. Because of the results obtained in this series of tests, it is now the practice to defat all digestion mixtures in which the fat content exceeds half the content of protein.

Some plant proteins contain considerable amounts of amide nitrogen. In Figure 2 it was demonstrated that ammonia reacts like a free amino group in the formol titration. Amide nitrogen, on the other hand, fails to influence the titration values. However, in the acid hydrolysis of the protein for the theoretical maximum formol titration, amide nitrogen is converted to ammonia. The possibility therefore existed that some error might be introduced unless correction is made for ammonia derived from this source. This was investigated and the results are presented in Table 6. Ammonia was determined by adjusting the pH of the suspension to 12.0 and vacuum-distilling the ammonia at 35 to 40° C. into solutions containing a known volume of standard acid. It will be noted that there was no difference in the rate or degree of hydroylsis of the protein as a result of free ammonia in the digest, the figures with and without corrections for ammonia being essentially the same. This is explained by the fact that during enzymic hydrolysis of the protein, ammonia was liberated at almost the same rate as the amino groups. Thus,

TABLE 6

Influence of Ammonia Derived from Amide Nitrogen on the In Vitro Test of Protein Digestibility

Sample (Protein Content)	Amide Nitrogen	Test	Hydrolysis of Proteins		
			After 1 Day	After 2 Days	After 5 Days
	% of total N		*percent*	*percent*	*percent*
Defatted Sunflower Meal (52.5%)	17	Direct	16	25	37
		Correcting for NH_3 in sples.	17	28	34
Yeast Powder (46.2%)	10	Direct	21	30	36
		Correcting for NH_3 in sples.	23	32	36
Dried Skim Milk (32.8%)	6	Direct	29	33	35
		Correcting for NH_3 in sples.	34	35	39
Hard Boiled Egg (12.0%)	6	Direct	11	16	31
		Correcting for NH_3 in sples.	10	14	31
Expeller Processed Soy Meal (49.0%)	1	Direct	14	25	37
		Correcting for NH_3 in sples.	14	25	37

although the actual titration values are somewhat different, particularly in the case of the sunflower meal where appreciable amounts of amide nitrogen are found, the over-all picture of digestion of the protein remains the same.

Another point worthy of mention is the effectiveness of the antiseptic in the enzymic digestion mixture. Toluene has been employed for this purpose in the current studies. Tests conducted on whole beef blood, a favorable medium for the propagation of many types of bacteria, have indicated that toluene is very effective as a bacteriostatic and even as a bactericidal agent. The use of toluene and benzene in microbiological assays when test materials are incubated with enzymes for the liberation of the bound forms of vitamins is, of course, common practice. When ineffective bacteriostatic agents were employed, putrefaction occurred in the digestion mixtures, and the formol titration values were as much as 30 percent greater than the figures for the acid hydrolyzed samples. This was due to acid decomposition products which are also measured in the formol titration.

With all the factors mentioned under control, Table 7 presents data on the reproducibility of the technique for estimating the susceptibility of proteins to *in vitro* enzymic digestion. For each experiment 200 mg. of USP pancreatin of the same batch was employed. These duplicate series of enzymic digestions were carried out at different periods, some spaced as much as one month apart. The data show that the reproducibility of the test procedure is excellent. Insofar as the formol titration itself is concerned, the precision attainable is similar to that of the usual acidimetric-alkalimetric titrations.

TABLE 7

*Reproducibility of Technique for Estimating Susceptibility of Proteins to Enzymic Digestion**

Sample	Protein Content (N x 6.25)	Degree of Hydrolysis of the Proteins		
		After 1 Day	After 2 Days	After 5 Days
	percent	*percent*	*percent*	*percent*
Casein	91.6	18	30	41
		19	32	43
Solvent Processed Soy Flour	52.7	8	9	11
		8	12	14
Expeller Processed Soy Meal	46.2	13	23	31
		14	21	33
Defatted Sunflower Meal	51.4	12	26	38
		16	25	37
Whole Oats	14.3	19	26	30
		17	22	28

* For each experiment 200 mg. of USP pancreatin of the same batch was employed.

In vitro digestibility studies

The first application of the *in vitro* protein digestibility test was made to soybean products which had undergone heat-processing to improve their nutritive value. This type of material is well suited for studying the validity of the hypothesis that rate of digestibility is an important factor in determining the utilization of the amino acids, since it shows no difference in amino acid composition or coefficient of digestibility before and after various types of processing, but exhibits marked differences in biological value. The results of two series of digestibility studies are presented in Table 8.

TABLE 8

Susceptibility of Soy Proteins to Enzymic Digestion

Sample (Protein Content)	Laboratory Processing	Hydrolysis of Proteins		
		After 1 Day	After 2 Days	After 5 Days
		percent	*percent*	*percent*
Solvent Processed Soy Flour (52.7%)	None	8	9	11
	△ 100° C. 7 days, atmospheric pressure	8	9	11
	△ in water, 100° C. 5 min., atmospheric pressure	7	9	11
	Autoclaved 10 min. at 5 lbs. pressure	9	10	14
	Autoclaved 10 min. at 10 lbs. pressure	12	21	24
	Autoclaved 30 min. at 10 lbs. pressure	14	23	29
Expeller Processed Soy Grits (48.7%)	None	16	20	24
	Autoclaved 10 min. at 10 lbs. pressure	20	23	29
	Autoclaved 30 min. at 10 lbs. pressure	22	27	33
	Autoclaved 30 min. at 15 lbs. pressure	18	25	34
	Autoclaved 45 min. at 15 lbs. pressure	16	25	33

Dry heating of soy flour at 100° C. at atmospheric pressure results in no improvement in the biological value of the protein. The same is generally conceded if the material is heated for a short period of time in boiling aqueous suspension. However, heating with superheated steam or autoclaving results in an increase in the biological value of the protein *(34, 62, 63, 49, 25, 77)*. In this table are presented the results of tests of two soy products, one a solvent-processed soybean flour, the other an expeller-processed soy grit. The solvent processing of soy flour, extraction with a fat solvent at moderately low temperatures (in the neighborhood of 58° C.) is not very effective in increasing the biological value of the protein. On the other hand, the expeller process for obtaining fat from soybeans involves the building up of a high internal temperature in the pack so that the protein receives effective heat treatment at the same time the fat is being expelled. It will be noted from the data in Table 8 that those conditions which fail to affect the biological value of the soy protein also fail to increase the digestibility of the soy protein. Thus, the solvent-processed soy when heated at 100° C. either in the dry state or in aqueous suspension exhibits no improvement in rate and degree of digestibility of the protein component. As the material is autoclaved there is a progressive increase in its digestibility concomitant with the increase in biological value. In the case of the soy grits improved biological value is accompanied by greater degree and rate of digestibility of the untreated sample. Here, too, an increase in digestibility of the protein occurs as a result of the autoclaving. This soon plateaus out, hence there is no advantage in autoclaving beyond a limited period or at increased pressure. Inasmuch as the availability of certain of the amino acids may be reduced by excessive heat treatment, it may be found that at some point before maximum digestibility is obtained the product possesses maximum biological value. Almquist and collaborators *(2)* and Hayward and his group *(32)* have demonstrated that the limiting amino acid in soy protein is methionine and that autoclaving increases the availability of this amino acid. But precautions must be taken lest the effort to increase the availability of methionine be accompanied by decreased availability of other amino acids, notably lysine.

In Table 9 are listed the results obtained in studies of the influence of processing on the susceptibility of other proteins to *in vitro* enzymic digestion. The marked increase in the digestibility of the protein in whole egg after heat coagulation is in agreement with the reports in the literature *(3, 12, 31)*. The protein in whole beef blood shows a similar improvement in digestibility; in the case of the milk proteins the same is noted but to a lesser degree. Heating whole oats under steam pressure appears to be without effect on digestibility. Tests conducted on samples autoclaved for shorter and longer periods of time gave essentially the same results as the untreated samples. In the case of the defatted wheat germ there is a decrease both in degree and in rate of enzymic hydrolysis of the protein after the autoclaving treatment. The significance of these changes on the nutritive value of the proteins can be ascertained only by correlation with animal assays. In view of species differences in biological responses, recently noted *(13)* in protein utilization studies, precautions must be observed in drawing broad generalizations.

TABLE 9

Influence of Processing on the Susceptibility of Proteins to Enzymic Digestion

Sample	Processing	Hydrolysis of Proteins		
		After 1 Day	After 2 Days	After 5 Days
		percent	*percent*	*percent*
Whole Egg	Raw	1	8	12
	Hard Boiled	11	16	31
Milk	None	19	24	28
	Skimmed and spray dried	29	33	35
Whole Oats	None	19	26	30
	Autoclaved 30 min. at 15 lbs. pressure	17	22	27
Defatted Wheat Germ	None	29	38	52
	Autoclaved 30 min. at 15 lbs. pressure	16	29	38
Beef Blood	None	4	8	16
	Proteins heat coagulated	31	47	60

Biological and Microbiological Studies of Food Proteins Improved in Nutritive Value by Heat-Processing

The remainder of this paper is concerned with animal and microbiological assays designed to test the validity of the hypothesis dealing with the importance of the rate of digestibility of proteins *in vivo*. The soybean has been selected as the prototype of those proteinaceous products whose nutritional value is improved by heat-processing.

In Table 10 is a summary of the data obtained in correlating the *in vitro* tests of protein digestibility with rat growth studies. The soy products, the control milk sample, and the bioassay values were kindly supplied by Dr. J. W. Hayward of the Archer-Daniels-Midland Company. He has expressed the relative protein efficiency of the soy samples as the weight gained per gram of protein consumed, assuming an efficiency of 100 percent for milk proteins. It will be observed that the extracted and *toasted* soy meal was superior to the expeller soy meal, which in turn was superior to the raw soy meal. His studies also demonstrated that these differences in protein efficiency were not correlated with the coefficient of digestibility of the proteins. It will be noted that the *in vitro* digestibility tests of the

TABLE 10

Correlation of the In Vitro Test of Protein Digestibility With Rat Growth Studies

Sample*	*In Vitro* Hydrolysis of Proteins			Protein Efficiency In Rat Assay†
	After 1 Day	After 2 Days	After 5 Days	
	percent	*percent*	*percent*	*percent*
Dried Skim Milk	29	33	35	100
Extracted and Toasted Soy Meal	17	34	46	86
Expeller Soy Meal	13	23	31	71
Raw Soy Meal	12	19	26	53

* Samples and bioassay values kindly supplied by Dr. J. W. Hayward of Archer-Daniels-Midland Co., Mineapolis. The meals were from the same batch of soybean.

† As weight gained per gram of protein consumed, expressed relative to the milk protein having an assigned value of 100%.

soy meals show the very same relationship as the animal assays. However, comparisons should not be made with the results obtained with the dried skim milk since the amino acid composition of the protein in the latter is different from that of soy protein. The *in vitro* digestibility data should be compared only in respect to an individual protein material to ascertain the influence of processing or storage for extended periods of time.

In Table 11 is a correlation of the results of *in vitro* tests of soy protein digestibility with nitrogen balance studies on rats, using for the latter the procedure of Mitchell and associates *(53)*. No. 6 was the raw soy meal; No. 5 the solvent-extracted meal; the others were heat-processed. All the meals were prepared from the same batch of soybean. It will be noted, in considering the data obtained with meals No. 1 through 6, that the decrease in rate and degree of protein digestibility *in vitro* is accompanied by a decrease in the biological value, while the coefficient of digestibility of the protein is not significantly affected. In other words, the heat-processing fails to make available (absorbable) a greater amount of protein nitrogen for the rat to use in the building of new tissue protein. However, the percentage of absorbed nitrogen retained for tissue protein anabolism was much greater in the case of the processed soy No. 1 as compared with the raw meal No. 6.[f] It would seem that the protein in soy meal No. 1, by virtue of being much more readily digestible, liberates methionine (the limiting amino acid) at a more rapid rate and at a more opportune time in relation to the other amino acids which it supplements. In the case of the raw soy, the methionine may be liberated too late to supplement the amino acids already absorbed. Thus, not only is the methionine inefficiently utilized for protein synthesis, but other amino acids, which require methionine supplementation, are likewise poorly utilized, being converted to energy rather than tissue substance.

On the other hand, it might be assumed that the coefficient of digestibility, dropping from 84 to 81, would

[f] The results with soy meal No. 7 are included in Table 13, although it is an exception to the general rule. The product was a heat-processed preparation, quite brown in color, with as high a biological value as soy meal No. 1, but it exhibited relatively poor digestibility *in vitro*. It may be that substances are present in this product as a result of the processing, inhibitory of enzymic activity *in vitro* but not *in vivo*.

TABLE 11

Correlation of the In Vitro Test of Protein Digestibility With Nitrogen Balance Studies on the Rat: Studies With Soy Protein

Soy Meal*	*In Vitro* Hydrolysis of Proteins			Rat Assays of Proteins	
	After 1 Day	After 2 Days	After 5 Days	Biological Value	Coefficient of Digestibility
	percent	*percent*	*percent*	*percent*	*percent*
1	14	25	37	71	84
2	13	22	33	67	85
3	14	22	32	66	82
4	11	17	23	64	84
5	10	16	21	61	81
6	11	16	22	53	81
7	11	15	24	72	88

* Sample No. 6 was the raw soy meal; No. 5 the solvent extracted meal; the others were heat-processed. All the meals were prepared from the same batch of soybean.

be of very great importance if the difference of 3 percent represented principally failure of methionine absorption. In that case, the differences in the biological values could be due simply to the degree rather than to the rate of methionine liberation *in vivo*. However, the hypothesis in the present study requires that the methionine in the raw soy be absorbed to almost the same extent as the methionine in the processed soy, but after too long a time interval effectively to supplement the other amino acids previously absorbed.

Because of these conflicting interpretations, specific tests were designed to study the problem. These involved studies of methionine absorption by the rat consuming the test rations. In conducting the nitrogen balances according to the technique described by Mitchell *(53)*, it is necessary to feed the animals a low protein diet containing lactalbumin, at a level of about 3 to 4 percent of the ration, before and after the actual assays with the test proteins. In the course of these successive assay periods a dry skim milk sample is included, so that comparisons may be drawn between the biological value and coefficient of digestibility of the test samples with that of whole milk protein. The results of the methionine balance studies are summarized in Table 12.

It will be noted that despite an increased methionine intake of 250 percent by the feeding of the milk proteins, the same fecal excretion of this amino acid occurred. This is good evidence that the 70 mg. of methionine

TABLE 12

Methionine Absorption by the Rat Consuming Diets Containing Milk or Soy Protein

Ration*		Intake of Methionine	Fecal Excretion of Methionine	Unabsorbed Methionine		Assay of Proteins	
Description	Protein Content			Corrected‡	Part of Intake	Biological Value	Coefficient of Digestibility
	percent	*mg.*†	*mg.*†	*mg.*†	*percent*	*percent*	*percent*
Lactalbumin	2.96	61	65	0	0	....	....
Lactalbumin	2.96	65	69	0	0	....	....
Dried Skim Milk	8.50	225	75	0	0	89	84
Processed Soy—1	9.06	161	146	76	47	71	84
Processed Soy—2	9.09	178	150	80	45	67	85
Processed Soy—3	9.10	141	129	59	42	66	82
Processed Soy—4	9.24	200	147	77	38	64	84
Extracted Soy—5	9.30	158	133	63	40	61	81
Raw Soy—6	8.99	154	145	75	49	53	81

* The soy meals were all from the same batch of soybean.

† Per rat test period of 7 days.

‡ Correction is made for the metabolic fecal methionine, 70 mg. per rat per period. The same group of six adult rats was employed throughout the balance studies.

excreted per rat during the 7-day test period represented metabolic fecal methionine and not methionine derived from dietary protein. The processed, extracted, and raw soy meals were all fed at a protein level of approximately nine percent. Correction of the total fecal methionine for the metabolic excretion of methionine gives an index of the quantity of this amino acid which failed to be absorbed. Expressing this in terms of methionine intake, it was found that as much as 38 to 49 percent of the methionine in soy products was not absorbed from the gastrointestinal tract of the rat. Furthermore, this absorption of methionine showed no correlation with the biological value. The values for unabsorbed methionine were essentially the same considering the assumption of a constant metabolic excretion. The differences in biological value are significant since the values were reproducible within several percent. Johnson, Parsons, and Steenbock *(39)* have reported negligible decreases in fecal sulfur and nitrogen when raw soy meal was replaced with a properly cooked product. However, greater retention (decreased urinary excretions) of both these factors was noted when the heat-processed soy meal was fed. These observations are in complete harmony with the hypothesis presented in this report since they, too, indicate no decrease in methionine absorption when the raw soy is ingested, but poor utilization of both this and the other amino acids. More recently, Evans *(19)* reported no correlation between the "digestible" organic sulfur content of soy protein and its nutritional value.

The present experiment constitutes a specific case where degree of digestibility of the protein in the gastrointestinal tract as well as the extent of absorption of the limiting amino acid, methionine, are unchanged, but nevertheless, the biological value (retention of absorbed nitrogen) varies considerably. Apparently the methionine in the raw meal is absorbed so late during the gastrointestinal journey that this amino acid, as well as the incompletely supplemented amino acids, are not efficiently utilized for the synthesis of body protein.

The validity of this interpretation is supported by further experimental findings, presented in Table 13. The test materials were the raw soy and the processed meal with the highest biological value. The biological values and coefficients of digestibility of both products are listed. The percent of dietary methionine unabsorbed is also indicated. According to the *in vitro* test, one is more readily digested than the other. The proteins of both samples were then analyzed for three amino acids, leucine *(70, 48)*, lysine *(16)*, and methionine *(47, 1)*. The values are essentially the same for the two products and agree with those reported in the literature for soy protein. The digests obtained, in the course of the *in vitro* enzymic hydrolysis of the protein, at the end of the first and fifth days were also subjected to amino acid analysis. For this purpose trichloroacetic acid was added to the digest to make a 7 percent concentration. The filtrates were then heated to convert the trichloroacetic acid to chloroform and carbon dioxide which were volatilized from the boiling solutions. These were then subjected to acid hydrolysis to convert peptides and possibly larger molecules to the free amino acids. Thus the values for leucine, lysine, and methionine in the filtrates of the protein digests include not only these amino acids in the free state but also in peptide linkage not precipitable by 7 percent trichloroacetic acid. Obviously the percentage amino acids so liberated would be considerably greater than that estimated by formol titration which is a measure of only the free amino groups.

Methionine was liberated very slowly from raw soy as compared to leucine, while lysine was most rapidly released by enzymic digestion. In the case of the processed soy, the same relationship held, although all amino acids were released at a somewhat faster rate. When the five-day digests were tested, it was found that almost all the lysine and more than half of the leucine had been liberated. In the hydrolysate of the raw soy meal only 36 percent of the methionine was found, while in the digest of the heat-processed meal there was 73 percent. These data indicate that methionine is liberated from soy proteins at a slower rate than the other two amino acids, and that heat-processing of this protein increases the rate of liberation of the methionine to a much greater extent than of leucine or lysine.

Biological and Microbiological Studies of Food Proteins Impaired in Nutritive Value by Heat-Processing

Earlier in this report it was pointed out that excessive heat-processing reduces the biological value of protein without affecting materially the protein content, the essential amino acid composition, or the degree of protein digestibility *in vivo*. In such cases lysine has been found to be the principal limiting amino acid. These findings are comparable to those noted with raw foods, improved in protein value by heat-processing, with the

TABLE 13

Rate of Enzymic Liberation of Amino Acids During the In Vitro Test of Protein Digestibility As A Critical Factor in Determining Nutritional Response

Experiment	Raw Soy Meal (No. 6)	Processed Soy Meal (No. 1)
Rat Assays		
Biological Value of the Proteins, %	53	71
Coefficient of Digestibility of the Protein, %	81	84
Dietary Methionine Unabsorbed, %	49	47
In Vitro Enzymic Hydrolysis of Proteins*		
After 1 day, %	11	14
After 2 days, %	16	25
After 5 days, %	22	37
Amino Acid Composition of the Proteins†		
Leucine, %	7.1	7.7
Lysine, %	5.1	5.4
Methionine, %	2.2	2.2
Liberation of Amino Acids‡		
Leucine	28	33
Lysine	45	56
Methionine	5	10
%, After 1 Day Hydrolysis		
Leucine	52	65
Lysine	77	84
Methionine	36	73
%, After 5 Days Hydrolysis		

* Based upon liberation of free amino groups, as determined by formol titration.

† Analyses on acid hydrolyzed samples, values calculated to 16% nitrogen.

‡ Includes not only free amino acids but also peptides, liberated during the *in vitro* enzymic hydrolysis and not precipitated by 7% trichloroacetic acid. The values listed are percentages of the total.

exception that methionine is the limiting amino acid in the raw products. This portion of the report is concerned with an investigation designed to determine whether the same mechanism is responsible for the reduced biological value of excessively heat-processed protein; namely, impairment in the susceptibility of the protein to enzymic digestion with the result that the limiting amino acid is released *in vivo* too late to supplement the amino acids already absorbed.

The results of tests with two samples of dried skim milk are of interest and are present in Table 14. These samples were obtained from the same processor at two periods one year apart. The amino acid composition was, of course, the same, and yet when tested on rats the samples showed an appreciable difference in biological value, 78 percent as compared to 90 percent, but no significant difference in coefficient of digestibility, that is, the fraction of dietary protein absorbed from the gastrointestinal tract. However, the more readily digestible sample, according to the *in vitro* test, exhibited the higher biological value.

In subsequent studies designed specifically to determine what mechanism is responsible for the poor utilization of lysine in excessively heat-processed protein, casein was selected as the test material. In confirmation of the work of others *(5, 17)*, it has been found (see Table 15) that dry-heating the sample [g] at elevated temperature (150° C.) does not destroy the limiting amino acid, lysine. When the samples were subjected to maximal enzymic hydrolysis (peptic followed by pancreatic digestion), it may be noted (see Table 16) that the degree of protein hydrolysis was not affected by the heat treatment and that the microbiologically available lysine *(72)* content was not substantially reduced.

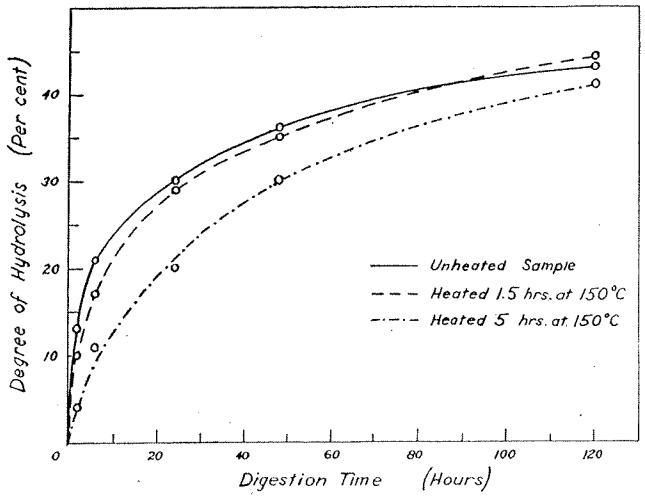


FIG. 3. Rate of *in vitro* pancreatic hydrolysis of casein samples. (From Pader, M., Melnick, D., and Oser, B. L. *J. Biol. Chem.*, 172, 763 [1948].)

In contrast to the above findings, striking differences in the *susceptibility* of the heated and unheated casein samples to *in vitro* pancreatic digestion are noted *(61)*. The data plotted in Figure 3 indicate that the heated protein samples are digested at significantly slower rates than the unheated control sample and that the rate of liberation of formol titratable nitrogen varies inversely with the processing time. The differences are of greatest relative magnitude during the early phase of hydrolysis. The ultimate degree of digestion of both the heated and unheated products eventually approaches the same value, in agreement with the results of the serial enzymic digestion experiments (see Table 16).

The differences in the *rate* of liberation of the available lysine from the casein samples are even more striking. Reference to Figure 4 shows that microbiologically available lysine was liberated from the heat-treated protein by pancreatin at a much slower rate than from the unheated product. The curves relating liberation of available lysine to time are linear up to a point at which they form a plateau. The relative slopes of the linear portions of the curves are as follows: 1, arbitrarily assigned to the control unheated sample; 0.66, casein heated 1.5 hours; and 0.25, sample heated 5 hours. Thus, substances with lysine activity are released from the unheated protein during the early period of digestion at a rate four times as great as from the sample submitted to prolonged heating, and 1.5 times as rapidly as from the intermediate preparation.

It is apparent that the differences in the content of microbiologically available lysine in the acid or final enzymic digests of heated and unheated casein are insufficient to account for the markedly reduced biological value of the heated protein. The results of the studies of

[g] The casein sample was obtained from the S. M. A. Corporation, Chagrin Falls, Ohio.

TABLE 14

Correlation of the In Vitro Test of Protein Digestibility With Nitrogen Balance Studies on the Rat: Studies With Milk Protein

Dried Skim Milk*	*In Vitro* Hydrolysis of Proteins			Rat Assays of the Proteins		
	After 1 Day	After 2 Days	After 5 Days	Series	Biological Value	Coefficient of Digestibility
	percent	*percent*	*percent*	*percent*	*percent*	*percent*
1	27	31	33	I	77	89
				II	79	87
2	32	36	41	I	90	85
				II	89	84

* Samples obtained from the same processor.

TABLE 15

The Lysine Content of the Test Casein Samples

Casein Sample	Lysine Found*
	percent
Unheated	8.1
Heated 1.5 hours at 150° C.	7.9
Heated 5 hours at 150° C.	7.6

* Determined microbiologically after complete acid hydrolysis; values calculated to the pure protein basis, 15.4 percent nitrogen.

TABLE 16

*Enzymic Liberation of Lysine for Microbiological Utilization**

Casein Sample	Degree of Hydrolysis**	Microbiologically Available Lysine†	Lysine Utilized‡
	percent	*percent*	*percent*
Unheated	57	6.5	80
Heated 1.5 hours at 150° C.	51	5.9	75
Heated 5 hours at 150° C.	57	5.2	68

* Peptic digestion followed by pancreatic digestion *(61)*.
** Estimated by formol titration.
† In the final enzymic digest, calculated to the original protein basis.
‡ Percent of total lysine in casein samples.

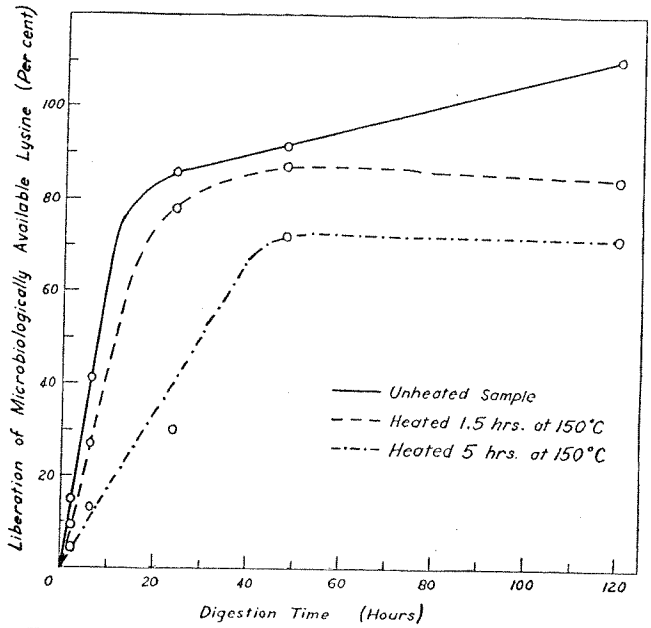


FIG. 4. Rate of *in vitro* pancreatic liberation of microbiologically available lysine from casein samples. (From Pader, M., Melnick, D., and Oser, B. L. *J. Biol. Chem.*, 172, 763 [1948].)

the rate of liberation of lysine for microbiological utilization provide a plausible explanation for the impairment of the biological value. The rate of release (and absorption) of lysine from heat-processed casein is too slow to permit effective supplementation of the other essential amino acids *in vivo* with the result that nitrogen retention for tissue protein synthesis is impaired; hence the need for supplementing the heated casein with lysine for optimal utilization. Indeed, from the work of others *(64)* the lysine in excessively heat-processed protein may be released so late during the gastrointestinal journey that a large fraction may escape absorption.

Biological Studies of Food Proteins Whose Nutritive Value Is Not Affected by the Heat-Processing Employed

The proteins in excessively heat-processed cereals are recognized to exhibit little nutritive value *(56, 44, 58, 71, 43, 64)*. However, when these are properly cooked no undesirable changes in the protein component occur. In fact the protein in the unheated products appear to be equal in nutritive value to those cooked for improved palatability *(65)*.

In studies, summarized in Table 17, of various oat samples(raw, pan-toasted, and thoroughly cooked at atmospheric pressure), no significant differences in the digestibility of the protein *in vitro* were noted. All four products were rapidly hydrolyzed and reached a maximum degree of digestibility according to this type of test. The lack of effect of the heat-processing on the digestibility of oat protein is also evidenced by the uniformity in its biological value as determined by the nitrogen balance experiments. The somewhat greater value of 81 percent for the whole oats may be attributed in part to a difference in the quality of the protein consumed since the protein in this sample comes from the bran as well as the endosperm.

TABLE 17

Correlation of the In Vitro Test of Protein Digestibility With Nitrogen Balance Studies on the Rat: Studies With Oat Protein

Oat Sample*	*In Vitro* Hydrolysis of Proteins			Rat Assays of Proteins	
	After 1 Day	After 2 Days	After 5 Days	Biological Value	Coefficient of Digestibility
	percent	*percent*	*percent*	*percent*	*percent*
Whole Oats	27	40	51	81	85
Hulled and "Pan Toasted"	25	34	44	74	87
Cooked	18	36	53	75	88
Cooked	20	40	47	79	89

* From the same batch of oats.

Summary and Conclusions

The concept of food protein as N × 6.25 should be extended to include a consideration of both the functional and nutritive properties of the protein. The functional properties, readily modified by heat-processing, affect appearance and palatability of the end-product and for this reason cannot be dissociated from the nutritive properties of the protein. The functional properties of the protein are usually evaluated by subjective utility tests; it has been suggested that these be supplemented with precise objective procedures for determining the degree of protein denaturation caused by the heat-processing.

The nutritive properties of protein may be determined by animal assays but here also the need to supplement the data with results of *in vitro* tests has been emphasized. Determinations of the essential amino acid composition of the protein and its susceptibility to enzymic digestion contribute to an explanation of results obtained by animal assays. A simple *in vitro* procedure for determining the susceptibility of protein to enzymic digestion has been presented in this report. It has been shown in studies with products, improved or impaired by heat-processing and exhibiting no change in amino acid composition or degree of digestibility, that rate of enzymic digestion is critical. For optimal utilization of food proteins all essential amino acids must not only be available for absorption but must be liberated during digestion *in vivo* at rates permitting mutual supplementation.

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